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Evaluation of pullulan production by a newly isolated *Micrococcus luteus*

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Pullulan is one of the most essential exopolysachharides (EPS) of α(Glucan) units in which most commonly α(1→4) linkage predominate. The *Aureobasidium pullulans* is the key microorganism for pullulan production. A major problem with *Aureobasidium pullulans* is coproduction of melanin along with pullulan. In this study, we looked for a novel strain having potential to produce pullulan without producing melanin and to evaluate various process parameters for its production. A total of 20 isolates were obtained from the soil sample but out of these only one strain that produced a significant amount of pullulan (71.39 mg/mL). This strain was identified as *Micrococcus luteus* (GenBank accession no KX261689) based upon the 16S rRNA sequencing. The characterization of pullulan was done with Enzymatic (Pullulanase) hydrolysis study and FTIR analysis. More than 85% hydrolysis of pullulan by pullulanase enzyme had also indicated the presence of α(1→6) and α(1→4) linkages in the structure of pullulan. Various evaluated values of different parameters for production of pullulan were found to be pH: 6.0, Temperature:35˚C, rpm: 250, Incubation time: 5 days. A Central Composite Design (CCD) was implemented in which peptone showed more effect than other factors in pullulan elaboration.

Keywords: Exopolysachharides (EPS), Pullulanase

The polysaccharides synthesized by the microbes have been used commercially with a wide range of applications. Pullulan is one of the most potent biocompatible polysaccharides which is basically synthesized by the *Aureobasidium pullulans*[1]. This polymer appears to be a linear α-glucan of maltotriose units with occasional branching of glucosyl or maltosyl substitution[2]. The regular alternation of α(1→4) and α(1→6) bonds results in structural flexibility and enhanced its hydrophilic nature.

Recently, many researchers have investigated that the surface modified pullulan with the substitution of different chemical groups have been used in various biomedical, pharmaceutical, and nutraceutical application[3,4]. The employment and application of pullulan in biomedical and tissue engineering field is emerging owing to its biocompatible, non-toxic, non-immunogenic and inert nature[5-7]. As a comparison to dextran, the degradation rate of pullulan in blood serum is much quicker[8]. It was found that, the degradation index was 0.7 for pullulan with the intervals of 48 h of incubation while it was of 0.05 for the dextran in the same condition[9]. The pullulan shows its resistance to the mammalian amylases, hence, it provides fewer calories and can be treated as dietary fibre. The solutions of the pullulan are of comparatively of very low viscosity, low consistency that resembles the Arabic gum[10,11]. The viscosity of pullulan solutions does not change in different conditions like pH changes, heating in different ranges of temperature and addition of most metal ions, including sodium chloride. Pullulan can be used as low-viscosity filler in beverages and in the preparation of sauces[12]. Pullulan has been widely used in lotions, cosmetics, and shampoos[13].

The melanin pigments are synthesized in the pentaketide pathway along with the pullulan. Hence, it is very difficult and time-consuming to difficult to extract pullulan out of melanin[14]. In the present research study, we have screened a pullulan producing new isolate of *Micrococcus luteus* from the soil sample in the agriculture field of Gatkesar area (17.4453° N, 78.6853° E) located, 25 km from the Hyderabad city, Telangana State, India. To the best of our knowledge, this is the first report for pullulan production by *Micrococcus luteus* without co-production of melanin pigments.

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Materials and Methods

Sample collection and screening

The soil samples from the crop field have been collected from Ghatkesar area, Hyderabad, India under aseptic condition using sterile gloves. Each sample was placed in a separate sterilized polypropylene bag and transported to the laboratory. These were subjected to serial dilution and were pour plated in the nutrient agar medium. In order to get a pure culture, the isolated colony was subjected to the streak plate method and was incubated at 30°C temperature for overnight. The microorganisms were maintained on nutrient agar slant containing peptone 5.0 g, yeast extract 2.0 g, NaCl 5.0 g, agar 15.0 g and distilled water 1000 L (pH 7.0). It was allowed to grow for 24 h at 30°C and then stored at 4°C. This was sub-cultured every two weeks.

Pullulan production and estimation

Inocula for pullulan production was prepared in two steps: in the first step, cells from fresh, nutrient agar cultures (24 h) were transferred to the tubes containing 7 mL of nutrient broth and the tubes were incubated at 30°C for 12 h at 200 rpm. In the second step, this culture was then transferred to 250 mL aliquots containing sterile 50 mL of nutrient broth and incubated at 200 rpm for 6 h at 30°C. The obtained inoculum (cell concentration approx. 10^8 cfu/mL) was used in pullulan production methodology. The composition of the medium for the production of pullulan was as follows: sucrose 60.0 g, K_2HPO_4 7.5 g, NaCl 1.5 g, $MgSO_47H_2O$ 0.4 g, yeast extract 0.4 g and distilled water 1000 mL[14,15]. The pH of the medium was adjusted to 6.5 by adding 0.1 M NaOH. After preparation and sterilization of the medium, 1 mL of the prepared inoculum was inoculated to 100 mL of the production medium and was incubated for 7 days under agitation (150 rpm) at 37°C. The recovery process for the pullulan production was carried out first by removing the cells by centrifugation at 7000 rpm, for 10 min followed by its precipitation with adding twice the volume of cold isopropyl alcohol. The pullulan was kept for drying at 70°C up to the attainment of constant weight. The percentage of pullulan was estimated as the grams of dry weight produced per 100 mL of fermentation broth[16].

Molecular characterization of the potential isolate

Identification of the bacterial culture was carried using 16S rRNA sequencing. Sequencing is a reliable method for the identification of bacteria species. Bacterial strain was grown in LB broth for 24 h, using AMpurE Bacterial gDNA Mini Spin kit, genomic DNA was extracted. Using universal PCR primer set for bacterial stains, forward primer 27F 5'AGAGTTT GATCCTGGCTCAG-3' and reverse primer 1492R-5'TACGGYTACCTTGTTACGACTT3''. Sequencing was carried out using universal sequencing primer set 518F-5'CCAGCAGCCGCGGTAATACG3' and 800R-5'TACCAGGGTATCTAATCC3' the 16S rRNA gene of the bacteria was sequenced at BioAxis DNA Research Center, Hyderabad. The program, Bio-Edit Sequence Alignment Editor Version 7.1.3.0 was run for the processing of sequence assembly. A sequence homo-similarity search was performed using the tools in NCBI BLAST (Link: http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned using the CLUSTAL W program[17]. For the neighbor-joining analysis distances between the sequences were calculated using Kimura's two-parameter model. The sequence was successfully submitted to nucleotide sequence database (GenBank) with accession no: KX261689.

FTIR analysis of pullulan

The characterization of purified pullulan was carried out using FT-IR spectroscopy (Model: ALPHA-T, Bruker) with the parameters 16 number of scans and $2cm^{-1}$ wave number. Dry precipitates were ground prior to the addition of 99% FT-IR grade potassium bromide (Sigma, USA). Commercial Pullulan (Molecular weight; 50 kDa, Sigma, USA) was used as a standard for the analysis[18].

Hydrolysis of pullulan using pullulanase

The purified pullulan from the *Micrococcus luteus* CM-01 strain was hydrolyzed by using pullulanase enzyme (Source: *Bacillus acidopullulyticus*, Sigma, USA). The reaction mixture (3 mL) consisting 0.5 mL of pullulan (0.4% w/v), 0.5 mL of diluted enzyme and 2.0 mL of 0.1 M phosphate buffer (pH 5.0) were taken in a test tube and incubated at 50°C with gentle agitation (150 rpm) for different time intervals (30-390 min)[16,19]. This reaction mixture was assayed for reducing sugars by the DNSA method[20] and the extent of hydrolysis in % was calculated as follows:

$$\text{Hydrolysis (\%)} = \frac{\text{Amount of reducing sugar released after the hydrolysis}}{\text{Amount of pullulan}}$$

Improvement of cultural conditions with evaluated physical parameters

Various optimal physical parameters required for maximal Pullulan production by *Micrococcus luteus* CM-01 in a shake flask system were studied. These

included different ranges of temperature (25, 30, 35, 40, 45 and 50°C), agitation speed (50, 100, 150, 200, 250 and 300 rpm), incubation time (1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th and 9th day), initial pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0). All experiments were carried out in triplicate and the values were reported as standard deviations.

Central Composite Design (CCD) with RSM model for optimization

Response surface methodology (RSM) was used to study the interaction among complex media components and their contribution towards pullulan production. The Central Composite Design (CCD) with 3 factors and 5 levels including 3 replicates were made for the establishment of second order 3D response surface with the help of Mini Tab 18 as given in Table 1. The CCD was developed as an embedded factorial matrix with center points and star points pointed (replicate of an axial point) around the center point which allows analyzing the curvature. The star points represent the extreme values (both low and high) for each factor in this design, and hence in case of full factorial design α is equal to $(2K)^{1/4}$. In this study K is equal to 3 i.e. sucrose, yeast extract and peptone, hence the value of α will be1.68179. Each variable was studied into five different levels and these are listed in Table 2. According to this, a set of 20 experiments which include 6 center points, 6 axial points with α value 1.68179 were carried out. A multiple regression analysis of the data was carried out and the second-order polynomial equation that defines predicted response (Y) in terms of independent variables was obtained:

$$Y = X_o + X_1A + X_2B + X_3C + X_{11}A_2 + X_{22}B_2 + X_{33}C_2 + X_{12}AB + X_{13}AC + X_{23}BC$$

Where, Y represents the response of variables, Xo is intercepting coefficient, X_1, X_2, X_3 are linear coefficients, X_{11}, X_{22}, X_{33} is squared coefficients and X_{12}, X_{13}, X_{23} are the interaction coefficients. Combination of factors (such as AB, AC, and BC) represents an interaction between the individual factors in that term.

Statistical analysis

Statistical analysis of the model was performed to evaluate the analysis of variants (ANOVA) after obtaining values of response by carrying out experiments as suggested by the model. Statistical significance of the model equation was determined using Fisher's test value (F-value) and proportion of variance explained by the model which was indicated by R^2 value. For each variable, the quadratic model was represented by contour plot and the 3-dimensional response surface plot was generated to understand the effect of variables individually and in combination. These plots were also used in determining the optimum media composition for obtaining higher production of pullulan. All the above statistical analysis was performed with the help of Mini Tab-Version 18.

Results and Discussion

Novel pullulan producing microorganism

A total of 20 strains were screened from different soil samples and were checked for the production of pullulan in the production medium. The results revealed that five strains were able to produce pullulan. The extracted and precipitated pullulan having the same types of alignment in the FT-IR as that of the standard commercial pullulan was taken into consideration for further investigation. The corresponding pullulan producing strain was

Table 1—Experimental range of the variables studied using CCD in terms of coded and actual factors

Variable	Symbol	Coded Level				
		–1.682	Lowv(-1)	Mid(0)	High(+)	+1.682
Sucrose	A	11.29	13	15.5	18	19.70
Peptone	B	0.32	1.00	2.00	3.00	3.68
Yeast extract	C	0.49	1.00	1.75	2.5	3.01

Table 2—Experimental designs used in RSM studies to understand interaction among media components for pullulan production

Std. Order	Run	Sucrose (g/L)	Peptone (g/L)	Yeast extract (g/L)	Observed response of pullulan (g/L)	Predicted response of pullulan (g/L)
1	2	18	1	1	22.71	21.62
2	16	15.5	2	1.75	48.12	50.79
3	15	15.5	2	1.75	57.44	55.81
4	7	13	3	2.5	48.5	50.61
5	11	15.5	0.31820	1.75	24.66	15.35
6	12	15.5	3.68179	1.75	71.39	69.27
7	19	15.5	2	1.75	58.27	56.79
8	17	15.5	2	1.75	57.61	56.79
9	13	15.5	2	0.488655	46.66	45.79
10	5	13	1	2.5	43.74	43.07
11	1	13	1	1	19.24	20.94
12	8	18	3	2.5	65.61	66.83
13	18	15.5	2	1.75	44.12	46.79
14	9	11.295517	2	1.75	49.34	47.05
15	14	15.5	2	3.011344	62.1	62.25
16	3	13	3	1	60	63.27
17	20	15.5	2	1.75	55.58	56.79
18	6	18	1	2.5	43.2	40.85
19	10	19.704482	2	1.75	53.28	50.85
20	4	18	3	1	65.78	69.38

designated as CM-01. Colonies of strain CM-01 showed mucous appearance on solid medium. The cells were gram-positive, coccus-shaped, and non-spore forming [21]. The assay for pullulan production was carried out with the addition of Isopropyl alcohol to the centrifuged culture filtrate of the fermentation broth and the pullulan was precipitated on the upper layer that has been recovered and kept for drying and was made to be powdered form.

FTIR analysis of EPS

All the peaks within the FTIR analysis have aligned in the same fashion without any shift for both the standard (Fig. 1A) i.e. Pullulan from Sigma, USA and test sample (Fig. 1B) i.e. pullulan extracted from *Micrococcus luteus* CM-01. The strong absorption at 3448 cm^{-1} indicates a number of the repeating units of –OH groups as in sugars. The other strong absorption at 2926 cm^{-1} confirms the presence of SP3-hybridization of C–H bond. Similarly, few significant peaks like 1,641 cm^{-1} for O–C–O bond, 1384 cm^{-1} for C–O–H bond and 992 cm^{-1} for C–O bond were found as in alkane compound. The FT-IR result confirms that the EPS synthesized by *Micrococcus luteus* CM-01 is primarily composed of pullulan. Some researchers had found a typical major broad stretching peak (3423 cm^{-1} for the hydroxyl group, and a weak band at 2925 cm^{-1} showing the C–H stretching vibration) of exopolysaccharides synthesized by *Micrococcus luteus*. They have also found the presence of uronic acid as denoted by the absorbance of 1739.5 cm^{-1} [21].

Hydrolysis of pullulan by using pullulanase

It is a well-known fact that pullulanase specifically hydrolyzes α(1→6) linkage of the linear α-D-glucan unit and releases maltotriose unit with one reducing

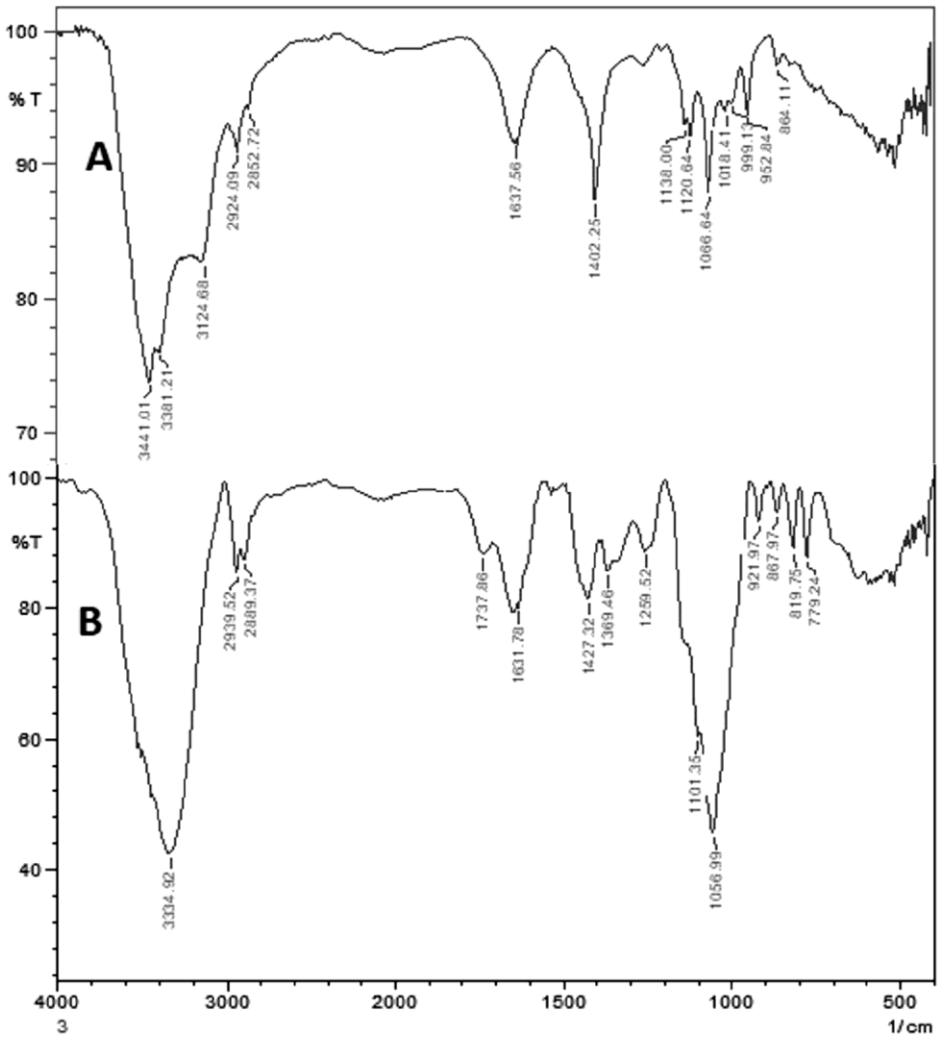

Fig. 1—Comparative Infra-red spectroscopy peaks of standard. (A) Pullulan from Sigma, USA and test sample; and (B) Pullulan from *Micrococcus luteus* CM-01

end of pullulan .The percentage of hydrolysis with the production of reducing sugar at 150 rpm in specific time intervals shows that maximum 85 % of pullulan hydrolysis was achieved after 390 min yielding 6.2 mg/mL of reducing sugars (Fig. 2). These results depict that the EPS used as a substrate for the pullulanase enzyme was identified as pullulan. Some researchers had also studied the hydrolysis pullulan by pullulanase enzyme. They found around 94.25% pullulan upon hydrolysis along with 3.77 mg/mL of reducing sugars. In our case, the hydrolysis percentage can be enhanced subjected to the use of purified pullulan[22].

Molecular characterization of isolates and phylogenetic study

The sequence was successfully submitted to nucleotide sequence database (GenBank) with Accession No: KX261689. The phylogenetic tree was constructed by running the CLUSTALW program with all the rRNA sequences having the most similarity value C 93% with the unknown sequence (Fig. 3).

Optimization of Physical parameters to improve the culture condition

The effect of temperature on pullulan production was investigated. It was found that maximum pullulan production was achieved at a temperature of 35°C (Fig. 4A). It also can be noticed that pullulan production decreased sharply when the fermentation temperature was higher than 35°C. This means that pullulan production by *Micrococcus luteus* was sensitive to higher temperature. In contrast, other reports have described optimal conditions for pullulan production at temperature 20 and 24°C. The different optimal temperature conditions reported in the literature may be also due to the differences in the types of strain, the composition of the fermentation medium, and culture conditions used[23,24].

The stirring speed is another important factor that contributes to the homogenization of the medium and oxygen transfer during fermentation process. Effect of this variable can be observed in Fig. 4B, with pullulan production being favored by high stirring speed, showing the necessity of higher oxygenation for the microorganism[25]. It was found that, maximum 45.34 mg/mL of pullulan was produced at 250 rpm and after that, the rate of production was decreased. In

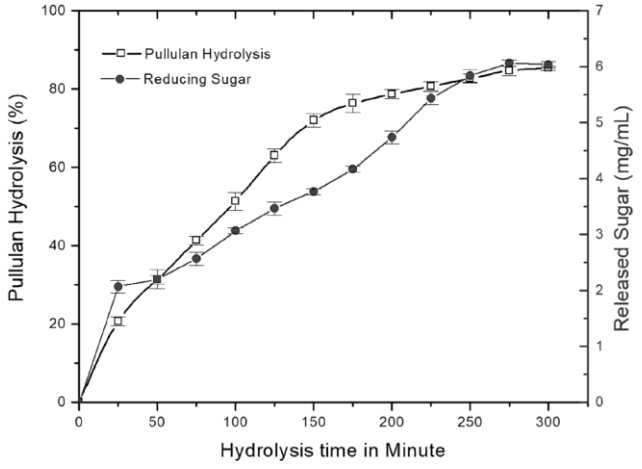

Fig. 2—Hydrolysis of pullulan obtained from *Micrococcus luteus* CM-01 strain by Pullulanase [Values are representative of three separate experiments]

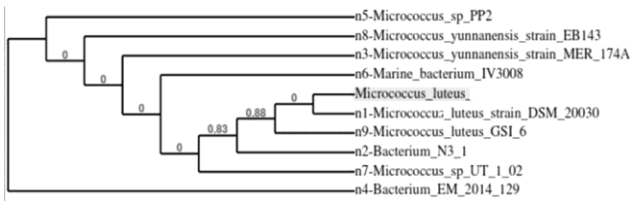

Fig. 3—Phylogenetic analysis of the strain CM-01 by Neighbor-joining method

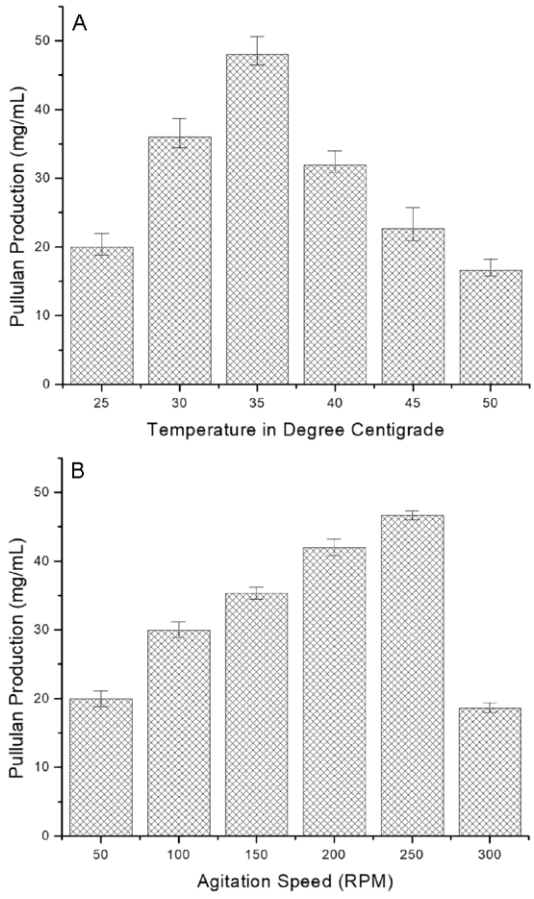

Fig. 4—Effect of (A) Temperature; (B) RPM; and (C) pH on pullulan production [Values are average of three replicates]

some cases, the changes in the morphological feature of the microorganism may influence the fermentative production of pullulan. The morphological changes are due to the induction of shear stress to the microbial cell. So in this case, when the agitation was increased (more than 250 rpm), due to more shearing force the cells got damaged and lost its efficiency.

The pH of fermentation media can influence the morphology of the microorganisms used, which may subsequently influence cell growth and pullulan production. Therefore, we have investigated the effects of different pH values ranging from 3.0 to 9.0 in the media on pullulan production by *Micrococcus luteus*. The maximum production of pullulan (46.33 mg/mL) was observed in the medium at an initial pH of 6.0 (Fig. 4C). In contrast to this result, several previous reports indicated that the optimal pH values for the pullulan production were obtained at initial pH of 5.0, 6.5 and 7.5[23,24,26]. The different optimum pH conditions reported in the literature may be due to the differences in the types of strain, the composition of the fermentation medium, and culture conditions used.

Experimental design and optimization with laboratory scale fermenter

The response surface methodology (RSM) and contour diagrams are the graphical representation of the regression equation developed. These plots were used to understand the media components, which are needed for significant production of pullulan. Therefore, three response surface graphs were obtained considering all possible combinations. The response surface plots and contour diagram obtained represents the interaction between sucrose (A) and peptone (B), at a specific concentration of yeast extract and also indicated their effect on maximum pullulan elaboration (Fig. 5 A and B). It was found that at low sucrose and peptone concentration the production of pullulan was very less and it increases gradually as the concentration of sucrose and peptone increases in the production medium. However, it was also noticed that a very high concentration of sucrose has a negative effect on pullulan elaboration. Maximum pullulan production in these specific conditions was found to be 71.39 mg/mL. It was found that a higher concentration of yeast extract was not favorable for pullulan production. The response for the interactive factors, of peptone (B) and yeast extract (C), when sucrose concentration was kept at a fixed value. From the RSM model, it was concluded that, at a lower concentration of peptone and yeast extract the pullulan production was very less. The standardized effect and main effect for pullulan production were also calculated (Fig. 5 C and D), where it was found that contribution of peptone is more as compared to other factors for pullulan elaboration. Some researchers had implemented response surface methodology in order to optimize the fermentation conditions for pullulan production by the strain *Aureobasidium pullulans* SK1002 in shaking flask cultures[27]. It was concluded that production of pullulan was significantly affected by temperature, fermentation time and initial pH. The application of response surface methodology resulted in a significant enhancement in pullulan production[28]. In some cases, Full Factorial Design (FFD) was implemented in the order to enhance molecular weight of pullulan biosynthesized by the strain of *Aureobasidium pullulans* SZU 1001 through Orthogonal Array method. The further yield of pullulan was enhanced with Central composite design[29].

Validation of shake flask experiments in a laboratory scale fermenter

A fermentation run was carried out in a 5 L batch fermenter (Lark Innovative Fine Teknowledge Company product) with 3 L working volume to validate the results obtained during optimization of the process in shake flask. From Fig. 6, it was found that the rate of pullulan production was directly proportional to the consumption of the carbon source. At the end of the 5th day of fermentation, 68.44 mg/mL of pullulan was produced, after which the pullulan produced in the fermentation medium was depleted.

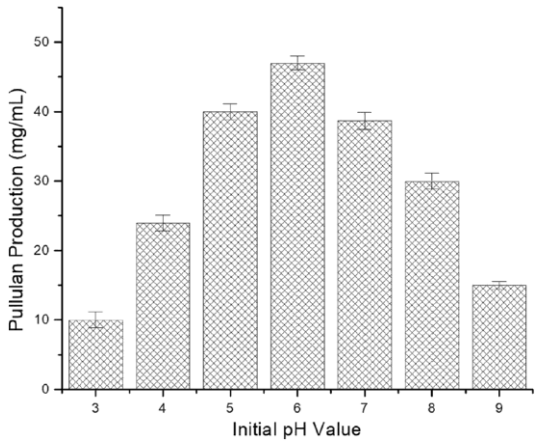

Fig. 5— Implementation of Central composite design for pullulan production. (A) Response surface methodology; (B) Contour plot; (C) Pareto chart of standardized effect for pullulan; and (D) Main effect plot for pullulan)

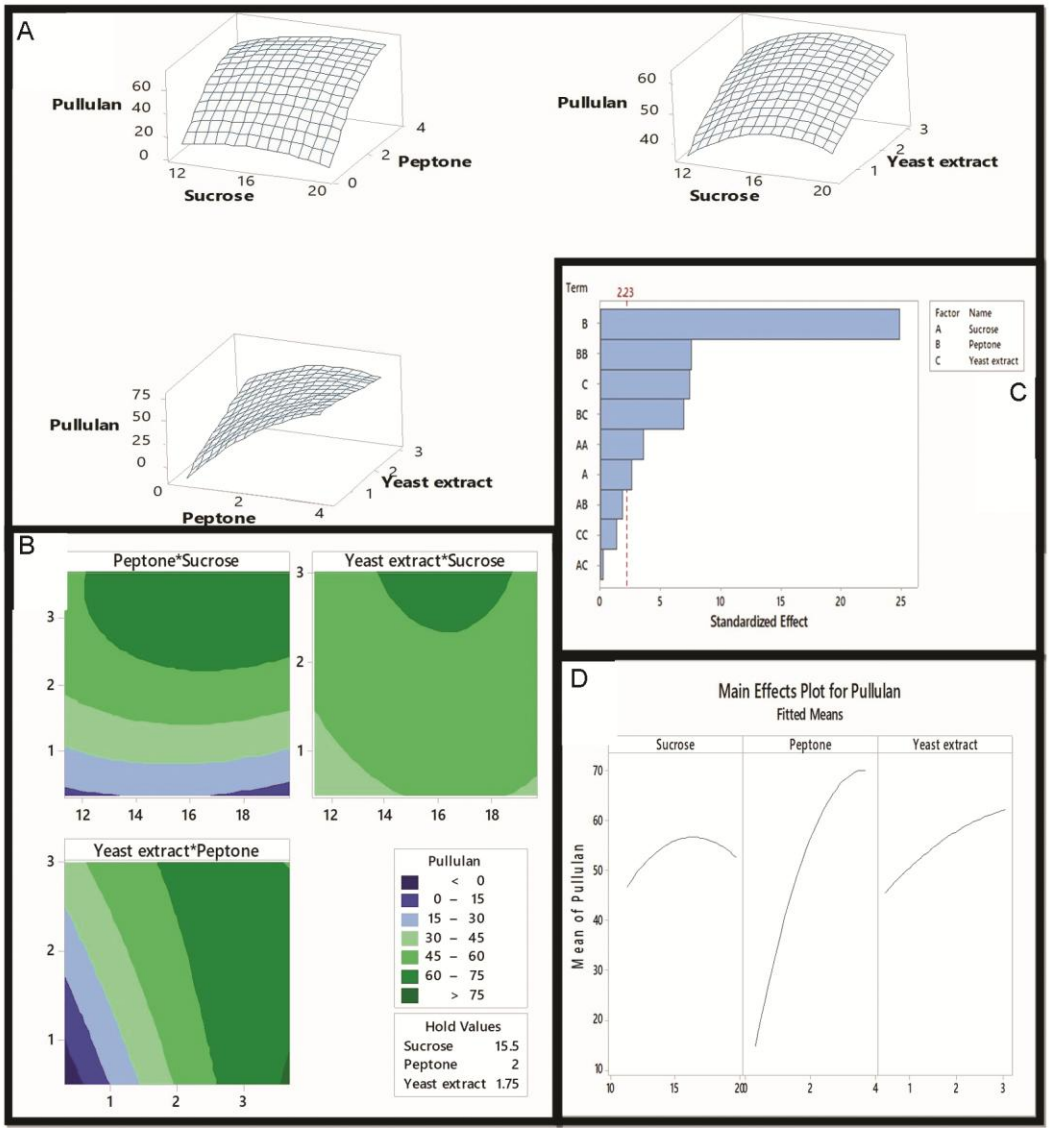

Fig. 6— Time duration of fermentation in an evaluated batch fermenter

This observation was also in agreement with the earlier observation made by some authors previously[30, 31]. This depletion was probably due to the fact that the carbon source in the medium was very less at the end of the fermentation. Hence, to sustain the growth and metabolic activity, the pullulan formed in the medium, getting degraded by the organism[32].

Conclusion

A non-pigmented pullulan producing a strain of *Micrococcus luteus* was evaluated for pullulan production successfully. FTIR and pullulanase hydrolysis confirmed basic identity of pullulan. Various physical parameters have been evaluated. From the CCD, it was found that peptone had more effect than other factors in pullulan elaboration. This study also indicated that, the process optimized in the shake flask level can be scaled up easily and it may lead to development of a successful cost effective technology for pullulan production.

Conflict of Interest

The authors declare that there is no conflict of interest.

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